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(54) Title: TRANSGENIC PLANTS WITH ENHANCED SULFUR AMINO ACID CONTENT

(57) Abstract

An enhanced seed content of sulfur-containing amino acids is achieved, without significant allergenicity, in transgenic plants, such as soybean plants, that contain a heterologous DNA segment embodying one or more copies of a gene that codes for a 10 kDa or 16 kDa rice prolamin or sunflower 2S albumin protein.

TRANSGENIC PLANTS WITH ENHANCED SULFUR AMINO ACID CONTENT

FIELD OF THE INVENTION

The present invention relates to transgenic plants that have elevated levels of sulfur-containing amino acids. More specifically, the invention relates to the production of transgenic plants that contain a DNA segment with a coding sequence corresponding to that of a gene for a sulfur-rich rice prolamin or sunflower 2S albumin protein.

BACKGROUND OF THE INVENTION

Many animal feed formulations are based on soybean or corn, which provides a concentrated source of protein. Soybean meal in particular lacks the essential sulfur-containing amino acids, methionine and cysteine, because the most abundant proteins accumulated in soybean seeds are deficient in these amino acids. Supplementation of animal feeds based on soybean or corn to bring the levels of essential sulfur-containing amino acids up to that required is a significant added expense.

Sulfur content of animal feed formulations based on soybeans has been increased by using soybeans transformed to express Brazil nut 2S albumin gene. The Brazil nut 2S albumin protein is a seed storage protein that is high in sulfur-containing amino acids, containing 18 mol per cent methionine and eight mol per cent cysteine. Since the Brazil nut 2S albumin is largely responsible for Brazil nut allergenicity, however, the use of seed from soybean plants transformed to express the Brazil nut 2S albumin gene is an unacceptable method of raising the sulfur content of animal feeds based on soybeans.

Brazil nut 2S albumin is a member of a superfamily of homologous proteins hypothesized to have a common ancestral gene(s). Kreis *et al.*, *J. Mol. Biol.* 183: 499 (1985). Many other seeds that have 2S albumin storage proteins are known to contain potent allergens. Melo *et al.*, *Food & Agricultural Immunology* 6: 185 (1994). This

is not surprising, given the degree of homology of 2S albumin storage proteins.

Monocot plants also contain seed storage proteins. The predominant storage proteins in most of the agronomically important cereals are the prolamins. Rice and oats are unusual cereal crops, in that the major storage protein is an 11S-type globulin, and only small amounts of prolamin proteins are found. Fewer monocot genes have been introduced successfully into dicot plants, for several reasons. There are definite differences in seed composition, gene regulation and post transciptional processing of storage proteins in dicots and monocots. In particular, monocot promoters typically do not function well in dicots. Other concerns relate to the stability of the sulfur-rich gene in the seed, and possible affects of the introduced gene on normal development of the plant. Altenbach *et al.*, *TIBTECH* 8: 156 (1990).

SUMMARY OF THE INVENTION

It is therefore an object of the present invention to provide plants that express an exogenous DNA sequence encoding a protein which is high in sulfur-containing amino acids but which does not cause an allergenic reaction in humans or animals.

It also is an object of the present invention to provide a method of increasing the content of sulfur-containing amino acids in an animal feed without supplementation.

It also is an object of the present invention to provide an approach for altering a plant so that it produces seeds characterized by levels of sulfur-containing amino acids that are higher than those of plants that have not been so altered.

In accomplishing these and other objectives, there has been provided, in accordance with one aspect of the present invention, a transgenic plant that contains a DNA molecule comprised of (A) a nucleotide sequence corresponding to a sulfur-rich rice prolamin gene or

sulfur-rich sunflower 2S albumin gene and (B) a promoter, operably linked to the nucleotide sequence, to effect expression thereof by the transgenic plant. The transgenic plant preferably is a corn or soybean plant. A preferred rice prolamin gene is a sulfur-rich 10 kDa rice prolamin gene, while a preferred sunflower 2S albumin gene is SFA-7 and SFA-8, preferably SFA-8. The invention also provides seeds produced by the transgenic plants.

A method of altering a plant to produce seed that contains levels of sulfur-containing amino acids that are higher than those of an unaltered plant is provided, comprising steps of (i) providing cells or tissues of a plant transformed with an expression vector that contains a nucleotide sequence corresponding to a sulfur-rich rice prolamin gene or sulfur-rich sunflower 2S albumin gene; (ii) propagating plants from the transformed cells or tissue; and (iv) selecting for further propagation those plants that produce sulfur-rich rice prolamin or sulfur-rich sunflower 2S albumin.

A method also is provided for increasing the content of sulfur-containing amino acids in an animal feed, preferably without supplementation, which comprises steps of (i) providing seeds from a plurality of plants, at least some of which plants contain a DNA molecule comprised of a nucleotide sequence corresponding to a sulfur-rich rice prolamin gene or sulfur-rich sunflower 2S albumin gene, operably linked to a promoter to effect expression of the nucleotide sequence by the plants; and (ii) processing the seeds into animal feed. The method results in a feed product that has not been supplemented by addition of sulfur-containing amino acids and that contains soybean or corn meal obtained from seed of transgenic plants.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples,

while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is the nucleotide and deduced amino acid sequence of sunflower 2S albumin 8 (SFA-8).

Figure 2 is the nucleotide and deduced amino acid sequence of the 10 kDa rice prolamin clone.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention allows for the production of transgenic plants that bear seeds characterized by a higher content of sulfur-containing amino acids, relative to plants that lack the heterologous DNA in question. Thus, an enhanced sulfur content in soybean (*Glycine max*) or corn (*Zea mays*), for example, is achieved, without significant allergenicity problems, by transforming soybean or corn material with a heterologous DNA segment that has a nucleotide sequence corresponding to one or more copies of a gene for a rice (*Oryza sativa* L) 10 kDa or 16 kDa prolamin or a sunflower (*Helianthus annus* L) 2S albumin protein, which are rich in sulfur.

In view of the similarity between the Brazil nut 2S albumin protein and its sunflower homolog, it is surprising that the sulfur-rich sunflower 2S albumin protein does not cause allergenicity problems. It also is surprising that a prolamin gene from rice, a monocot, can be successfully expressed in soybean, a dicot, without stability problems.

Sulfur-rich sunflower 2S albumin proteins are described, by example, by Lilley *et al.*, "Isolation and Primary Structure for a Novel Methionine-rich Protein from Sunflowerseeds (*Helianthus annus*. L)," *in* PROCEEDINGS OF THE WORLD CONGRESS ON VEGETABLE PROTEIN UTILIZATION IN HUMAN FOODS AND ANIMAL FEEDSTUFFS 497-502 (1989), and by Kortt *et al.*, *Phytochemistry* 29: 2805 (1990), the respective contents of which are incorporated herein by reference.

Eight proteins, denoted sunflower albumins (SFA) 1 to 8, are identified in the 2S albumin fraction. Two of these, SFA-7 and SFA-8, are sulfur-rich. They contain about eight mol per cent cysteine and 16 mol per cent methionine. SFA-8 is present in greater amounts than SFA-7, and for this reason is preferred. The nucleotide sequence of SFA-8 is shown in Figure 1.

Matsumura *et al.*, *Plant Molec. Biol.* 12: 123 (1989), describe three prolamin polypeptides isolated from rice seeds, denoted "10 kDa," "13 kDa" and "16 kDa." The 10 kDa and 16 kDa proteins are said to be sulfur-rich. A full-length clone for the 10 kDa prolamin also is described, and this embodiment is preferred for use according to the present invention. The nucleotide sequence of the 10 kDa clone is shown in Figure 2.

In accordance with the present invention, a DNA molecule comprising a transformation/expression vector is engineered to include a sequence from one of the sulfur-rich sunflower 2S albumin genes or from the 10 kDa or 16 kDa sulfur-rich rice prolamin gene. Isolation and cloning of these genes by standard methodology is described in Lilley *et al.*, Kortt *et al.* and Matsumura *et al.*, *supra*. The rice prolamin genes first may be modified to reflect preferred codon usage in dicots, prior to introduction into soybean.

The sulfur-rich sunflower 2S albumin genes and the 10 kDa or 16 kDa sulfur-rich rice prolamin gene of the present invention can be isolated from a natural source or synthesized from a known sequence. The sequence may be obtained by making a cDNA from mRNA. The sequence may also be derived from a genomic DNA sequence. The sequence may be subcloned in a vector of choice. When isolating the sulfur-rich sunflower 2S albumin or sulfur-rich 10 kDa or 16 kDa rice prolamin gene from the genome, or subcloning these genes, the methodologies used would include identification of the gene by hybridization with probes, PCR, probe/primer/synthetic gene synthesis,

sequencing, molecular cloning and other techniques which are well-known to those skilled in molecular biology.

Once the gene of choice has been isolated, a copy of its sequence is placed into an expression vector by standard methods. For a general description of plant expression vectors, see Gruber *et al.*, "Vectors for Plant Transformation," *in* METHODS IN PLANT MOLECULAR BIOLOGY AND BIOTECHNOLOGY 89-119 (CRC Press, 1993). It is preferred to insert multiple copies of an expression cassette containing the gene to be introduced into an expression vector, preferably at least four copies. The selection of an appropriate expression vector will depend upon the method of introducing the expression vector into host cells.

A typical expression vector contains: prokaryotic DNA elements coding for a bacterial replication origin and an antibiotic resistance gene to provide for the growth and selection of the expression vector in the bacterial host; a cloning site for insertion of the exogenous DNA sequence; eukaryotic DNA elements that control initiation of transcription of the exogenous DNA sequence, such as a promoter and an optional enhancer; and DNA elements that control the processing of transcripts, such as a transcription termination-polyadenylation sequence. The vector also could contain additional sequences that are necessary to allow for the eventual integration of the vector into a chromosome.

Expression of the gene sequence is under the control of a promoter. Examples of suitable promoters are the promotor for the small subunit of ribulose-1,5-bis-phosphate carboxylase, promoters from tumor-inducing plasmids of *Agrobacterium tumefaciens*, such as the nopaline synthase and octopine synthase promoters, and viral promoters such as the cauliflower mosaic virus (CaMV) 19S and 35S promoters or the figwort mosaic virus 35S promoter. The promoter can be constitutive or inducible.

Especially preferred is a "seed tissue-preferred" or "seed tissue-specific" promoters, that is, promoters that drive high expression of the heterologous DNA segment in seed tissue where control of genes that are involved in seed metabolism is desired, and little or no expression in other parts of the plant. Manufacture of sulfur-rich proteins in other parts of the plant needlessly expends the plant's energy. Examples of known seed tissue-preferred or seed tissue-specific promoters include the soybean promoter of ß-conglycinin, also known as the 7S protein, which drives seed-directed transcription (Bray, *Planta* 172: 364 (1987)) and seed-directed promoters from the zein genes of maize endosperm (Pedersen *et al.*, *Cell* 29: 1015 (1982)). Dicot promoters are preferred for use in soybeans, and a particularly preferred promoter is the bean phaseolin promoter.

In addition to a suitable promoter, one or more enhancers are useful in the invention to increase transcription of the introduced DNA segment. The enhancer or enhancer-like element can be inserted into the promoter to provide higher levels of transcription. Examples of such enhancers include *inter alia*, viral enhancers like those within the 35S promoter, as shown by Odell *et al.*, *Plant Mol. Biol.* 10: 263-72 (1988), and an enhancer from an opine gene as described by Fromm *et al.*, *Plant Cell* 1: 977 (1989).

Selectable marker genes, in physical proximity to the introduced DNA segment, are used to allow transformed cells to be recovered by either positive genetic selection or screening. The selectable marker genes also allow for maintaining selection pressure on a transgenic plant population, to ensure that the introduced DNA segment, and its controlling promoters and enhancers, are retained by the transgenic plant.

Many of the commonly used positive selectable marker genes for plant transformation have been isolated from bacteria and code for enzymes that metabolically detoxify a selective chemical agent which may be an antibiotic or

a herbicide. Other positive selection marker genes encode an altered target which is insensitive to the inhibitor.

A preferred selection marker gene for plant transformation is the neomycin phosphotransferase II (*nptII*) gene, isolated from Tn*5*, which confers resistance to kanamycin when placed under the control of plant regulatory signals. Fraley *et al.*, *Proc. Nat'l Acad. Sci. USA* 80: 4803 (1983). Another useful selectable marker is the hygromycin phosphotransferase gene which confers resistance to the antibiotic hygromycin. Vanden Elzen *et al.*, *Plan Mol. Biol.* 5: 299 (1985). Additional positive selectable markers genes of bacterial origin that confer resistance to antibiotics include gentamicin acetyl transferase, streptomycin phosphotransferase, aminoglycoside-3'-adenyl transferase and the bleomycin resistance determinant. Hayford *et al.*, *Plant Physiol.* 86: 1216 (1988); Jones *et al.*, *Mol. Gen. Genet.* 210: 86 (1987); Svab *et al.*, *Plant Mol. Biol.* 14: 197 (1990); Hille *et al.*, *loc. cit.* 7: 171 (1986).

Other positive selectable marker genes for plant transformation are not of bacterial origin. These genes include mouse dihydrofolate reductase, plant 5-enolpyruvylshikimate-3-phosphate synthase and plant acetolactate synthase. Eichholtz *et al.*, *Somatic Cell Mol. Genet.* 13: 67 (1987); Shah *et al.*, *Science* 233: 478 (1986); Charest *et al.*, *Plant Cell Rep.* 8: 643 (1990).

Another class of useful marker genes for plant transformation with the DNA sequence requires screening of presumptively transformed plant cells rather than direct genetic selection of transformed cells for resistance to a toxic substance such as an antibiotic. These genes are particularly useful to quantitate or visualize the spatial pattern of expression of the DNA sequence in specific tissues and are frequently referred to as reporter genes because they can be fused to a gene or gene regulatory sequence for the investigation of gene expression. Commonly used genes for screening

presumptively transformed cells include β-glucuronidase (GUS), β-galactosidase, luciferase, and chloramphenicol acetyltransferase. Jefferson, *Plant Mol. Biol. Rep.* 5: 387 (1987); Teeri *et al.*, *EMBO J.* 8: 343 (1989); Koncz *et al.*, *Proc. Nat'l Acad. Sci. USA* 84: 131 (1987); De Block *et al.*, *EMBO J.* 3: 1681 (1984). Another approach to the identification of relatively rare transformation events has been use of a gene that encodes a dominant constitutive regulator of the *Zea mays* anthocyanin pigmentation pathway. Ludwig *et al.*, *Science* 247: 449 (1990).

In order to create an expression vector containing the gene or genes of interest, an expression cassette first is made by inserting a cloned sunflower 2S albumin or rice prolamin gene into a plasmid under the control of a regulatory sequence. The resulting expression cassette can be ligated back to itself to produce an expression cassette with a tandem repeat of the cloned gene. A further ligation can be performed to generate a construct that contains four tandem copies of the gene. One or more copies of the expression cassette containing the introduced DNA segment corresponding to the sulfur-rich 2S albumin sunflower gene or sulfur-rich 10 kDa or 16 kDa rice prolamin gene then is transferred to an expression vector. In a preferred embodiment, the vector also contains a gene encoding a selection marker which is functionally linked to promoters that control transcription initiation. Preferably, a disarmed Ti-plasmid is used as a vector for foreign DNA sequences.

To create a transgenic plant, an expression vector containing a sulfur-rich 2S albumin sunflower gene or sulfur-rich 10 kDa or 16 kDa rice prolamin gene can be introduced into protoplasts; into intact tissues, such as immature embryos and meristems; into callus cultures or into isolated cells. Preferably, expression vectors are inserted into intact tissues, particularly explants derived from hypocotyl or cotyledonary nodes of a germinated seed. In this regard, an explant is a piece

of tissue that is taken from a donor plant and is capable of producing callus in culture. Hypocotyl tissue is that portion of the stem of a plant embryo or seedling below the cotyledons and above the root. A cotyledon is an embryonic leaf, and a cotyledonary node is that part of the seedling between the embryonic axis and the cotyledons which botanically defines the division of the hypocotyl and the epicotyl, or embryonic shoot. General methods of culturing plant tissues are provided, for example, by Miki *et al.*, "Procedures for Introducing Foreign DNA into Plants," *in* METHODS IN PLANT MOLECULAR BIOLOGY AND BIOTECHNOLOGY 67-88 (CRC Press 1993).

A preferred method for introducing an expression vector into plant tissue is direct infection or co-cultivation of plant tissue with *A. tumefaciens* that contains an expression vector with the gene of interest and associated regulatory elements. Horsch *et al.*, *Science* 227: 1229 (1985). A preferred expression vector is the vector pARC12 (p1830), a plasmid which is part of a binary Ti plasmid system of *A. tumefaciens* and which contains nopaline synthase/neomycin phosphotransferase II (NPTII) as a promoter and selectable marker for transformed plant cells.

The expression vector preferably is transformed into *A. tumefaciens* using a freeze-thaw technique, as described in PLANT MOLECULAR BIOLOGY (Gelvin and Schilperoort, eds., Kluwer Academic Publishers, 1988).

After an *A. tumefaciens* strain is confirmed to contain a complete copy of the construct, the construct can be transferred to the target plant. A suitable model for demonstrating transformation of a plant by the sulfur-rich sunflower 2S albumin and sulfur-rich rice prolamin genes is *Agrobacterium*-mediated transformation of the tobacco plant, *Nicotiana tabacum*. In this model, an *Agrobacterium* strain confirmed to contain the construct is used to inoculate wounded tobacco plants to generate the transgenic events. Spielmann *et al. Mol. Gen. Genet.* 205: 34 (1986).

When *Agrobacterium* is used to transfer an expression vector to soybean, induction of the virulence (*vir*) genes in *Agrobacterium* leads to enhanced transformation. Cultured soybean cells lack or have a limiting amount of the necessary signal molecules to initiate the transformation process, and induction of the *vir* genes is necessary to achieving successful transformation. Various physical parameters can be used to induce the *vir* genes. Various compounds can be used, individually or in combination, to induce the *vir* gene. Exemplary of such compounds are phenolic compounds such as acetosyringone, α-hydroxyacetosyringone, acetovanillone, syringaldehyde, syringic acid and sinapinic acid.

Low temperatures and low pH during co-cultivation also lead to improved transformation, presumably as a result of an effect on the *vir* genes. Temperatures below 26° result in more effective transformation, and a temperature of about 20° is preferred. Buffering at a pH below 6.0, preferably a pH of about 5.5, also improves transformation efficiency.

Transformation of soybean by *Agrobacterium* also is dependent upon concentration of bacteria in the inoculum. In general, higher numbers of bacteria result in more transformation events. Preferably an inoculation period of at least 30-minutes, and a concentration of bacteria of at least $3x10^8$ viable cells/ml are used.

For transformation of corn, techniques other than *Agrobacterium*-mediated transformation are preferred. An especially preferred methodology in this regard entails subjecting an exposed corn meristem to biolistic bombardment, in order to target non-differentiated meristem cells for transformation, as described in international application PCT 96/04392. According to this approach, meristem tissue is manipulated following bombardment in order to enlarge transgenic sectors, either through selection and/or through effecting a proliferation from the tissue of shoots or multiple meristems *per se*. The shoot population thus obtained

then is screened, by means of a nonlethal enrichment assay, to identify either chimeric sectors that will contribute to germline transmission, or non-sectored, periclinal chimeras that will by definition transmit to progeny. Increased time in culture, under selection, enhances the prospects for sectoral-to-periclinal conversions, and also selects for L1-to-L2 conversions which, through a shift in position, ultimately contribute to the germline.

After screening, transgenic plants are established from transformed explants by conventional techniques known to the skilled artisan. A preferred technique is cultivation of transformed explants in liquid counterselection medium, followed by transfer to solidified selection medium. The process is repeated as described in the art for identification by transgenic markers such as β-glucuronidase (*gus*). McCabe *et al.*, *Bio/Technology* 6: 922 (1988). Shoots are induced in transgenic explants by known methods. Wright *et al.*, *Plant Cell Rep.* 5: 150 (1986); Barwale *et al.*, *Planta* 167: 473 (1986). The shoots are excised and, by the addition of pyroglutamic acid to a hormone-enriched growth medium, roots readily are induced. Whole mature, reproductive plants are produced after transfer to greenhouse culture in soil.

Seed from transgenic plants according to the invention contain significant levels of sulfur-containing amino acids, particularly methionine. Expression of the sulfur-rich genes was accompanied by a concomitant decrease in trypsin and chymotrypsin inhibitor activities. These endogenous inhibitors are relatively rich in sulfur-containing amino acids, and the overexpression of the introduced genes may be depleting the normal free sulfur pools in the seed, with the result that sulfur is being scavenged from endogenous proteins. The transgenic plants appear to accumulate the heterologous proteins at the expense of certain endogenous proteins to maintain a homeostatic condition.

The changes produce no apparent effect on the viability of the transgenic plant or on the total seed protein.

Analysis of seed proteins from transgenic plants reveals no larger precursor proteins, suggesting that the transgenic protein is being processed completely, as it would be in the plant of origin. Amino acid analysis of seeds from transgenic plants shows no significant changes as compared to controls, except for increased levels of methionine.

While the present invention has been described in detail with respect to particular preferred embodiments, it should be understood that such description is presented by way of illustration and not limitation. Many changes and modifications within the scope of the present invention may be made without departing from the spirit thereof, and the invention includes all such modifications. Thus, while *A. tumefaciens* is a preferred vector, other types of vectors can be used for transformation by procedures such as direct gene transfer, as described, for example, in PCT application WO 85/01856 and in European application 0 275 069; *in vitro* protoplast transformation, which is the subject of U.S. patent No. 4,684,611, for instance; plant virus-mediated transformation, illustrated in European application 0 67 553 and U.S. patent No. 4,407,956; and liposome-mediated transformation according to U.S. patent No. 4,536,475, among other disclosures. Direct transfer methods also may be employed, such as microprojectile-mediated delivery, DNA injection, and electroporation. See, for example, Gruber *et al.* and Miki *et al.*, both cited above, and Klein *et al.*, *Bio/Technology* 10: 268 (1992).

The present invention is further described by reference to the following, illustrative example.

EXAMPLE: *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF TOBACCO OR SOYBEAN TO PRODUCE A TRANSGENIC PLANT THAT HAS A HIGH CONCENTRATION OF SULFUR-CONTAINING AMINO ACIDS

A. Creation of expression vector that contains sulfur-rich sunflower 2S albumin and rice prolamin genes

Full-length cDNA clones for the 10 kDa rice prolamin and sunflower SFA-8 proteins were obtained using RT-PCR with first strand cDNA as template and gene-specific primers designed against published sequences. See Matsumura *et al.* and Lilley *et al.*, *supra*. The resulting PCR products were subcloned into pBluescript SKII and confirmed by sequence analysis. The genes were transferred into p4752, which contains the 5' and 3' regulatory sequences from phaseolin. Sengupta-Gopalan *et al.*, *Proc. Nat'l Acad. Sci. USA* 82: 3320 (1985). The resulting expression cassettes contained SFA-8 (p6445) and 10 kDa rice prolamin (p6465) under control of the phaseolin regulatory sequences. p6445 and p6465 were then ligated back to themselves to produce p6668 and p6670, respectively, which contained tandem repeats of the expression cassettes. A final set of ligations was performed with p6668 and p6670 to generate p7518 and p7519, respectively, constructs which contain four tandem copies of the expression cassettes.

The constructs containing the four tandem copies were then transferred to the binary vector pARC12 (p1830), a vector which contains the NPTII selectable marker for plant selection. The resulting binary vectors for 4X SFA-8 and 4X 10 kDa rice prolamin are denoted p6704 and p7499, respectively. These vectors were transformed into *Agrobacterium tumefaciens* strain LBA4404 by a freeze-thaw method (PLANT MOLECULAR BIOLOGY, *op cit.*), and presence of a complete copy of the 4X construct was confirmed.

B. Explant preparation, transformation and transgenic plant recovery

Tobacco

Tobacco plants (*Nicotiana tabacum*) var. Xanthii, were cultured from 1 cm apical or axillary explants on OMS, according to Murashige *et al.*, *Physiologia Plantarum*, 15:473 (1962), in a Magenta Box (Magenta Corp., Chicago, Ill.) at 27°C with a 16 hour light/8 hour dark photoperiod. The first four fully expanded leaves below the apex (leaves 3, 4, 5 or 6) were removed. Leaves were placed, one at a time, in a 100 mm petri dish with several Whatman #1 filter discs, 7cm, soaked with liquid medium. A sterile, sharp, #2 cork bore (0.5 cm i.d.) was used to punch leaf discs, avoiding the midrib and veins. Discs were held on filter paper at 100% humidity in a separate petri dish with Whatman filters and liquid medium.

Overnight cultures of *A. tumefaciens* LBA4404 harboring p6704 and p7499, respectively, were grown to log phase in Minimal A medium containing tetracycline, 1.0 μg/ml. Cultures were pooled and an optical density at 550 nm was measured. Culture density was adjusted by dilution with liquid medium to about 5×10^5 bacteria/ml.

Leaf discs were inoculated by dipping them individually in a suspension of *Agrobacterium* harboring the 4X construct. Each disc was held submerged in the suspension for 0.5 -1.0 second, to ensure that all edges were infected. Each disc then was placed on a filter on cocultivation medium the induces regeneration of shoots in a petri dish. The medium was supplemented with phytohormones and included major salts and MS micronutrients (Murashiga, *op. cit.*) supplemented with sucrose, 3.0% w/v; naphthalene acetic acid (NAA), 0.1 mg/L and 6-benzylaminopurine (BAP), 1.0 mg/L. The medium was buffered to pH 5.7. The filter was dry enough to wick away excess inoculum. Ten to twelve discs per 7 cm filter per 100 mm plate were plated. The petri dishes

were covered with parafilm and kept in low light at 28°C for two days.

After two days the explants were moved to a liquid counterselection medium, a medium having the same basic composition as that used during cocultivation, but with the addition of cefotaxime, 500 μg/ml and vancomycin, 100 μg/ml. The discs were washed with gentle continuous gyratory shaking for 3-6 hours with at least one change of liquid medium.

The discs then were placed on solid medium, a medium having the same basic composition as that used during cocultivation, but with the addition of vancomycin, 100 μg/ml and carbenicillin, 500 μ/ml. Excess liquid was evaporated before plating ten to twelve discs per 100 mm plate. The explants were cultures for 3 days at 28°C in low light.

Discs were transferred to solid selection medium, a medium having the same basic composition as that used during cocultivation, but with the addition of vancomycin, 100 μg/ml; carbenicillin, 500 μg/ml; and kanamycin, 100 μg/ml. The discs were cultured at 26°C in high light with 16 hour light/8 hour dark photoperiod.

Transformed sectors became visible approximately two weeks later. Shoots appeared soon after callus and these were removed from the callus and place on hormone-free medium for rooting.

Soybean

Seeds of soybean (*Glycine max*), var. PHI9341, were surface sterilized by exposure to chlorine gas evolved in a glass bell jar. Gas was produced by adding 3.5 ml hydrochloric acid (34-37% w/w) to 100 ml to sodium hypochlorite (5.25% w/w). Exposure was for 16-20 hours in a container approximately one cubic foot in volume. Surface sterilized seed was stored in petri dishes at room temperature. Seed was germinated by plating of 1/10 strength agar solidified medium according to Gamborg (B5 basal medium with minimal organics, Sigma Chemical Co., cat. no. G5893, 0.32 gm/L; sucrose, 0.2% w/v and

2-[N-morpholino]ethanesulfonic acid] (MES), 3.0 mM without plant growth regulators and culturing at 28°C with a 16 hour day length and cool white fluorescent illumination of approximately 20 μEm^2S^1. After three or four days, seed was prepared for co-cultivation. The seed coat was removed and the elongating radicle was removed 3-4 mm below the cotyledons. Ten prepared seed were held in each of several petri dishes.

Overnight cultures of *A. tumefaciens* LBA4404 harboring p6704 and p7499, respectively, were grown to log phase in Minimal A medium containing tetracycline, 1.0 μg/ml. Cultures were pooled and an optical density at 550 nm was measured. An amount of culture sufficient to collect upon sedimentation between 1.0 and 2.0 x 10^{10} cells, where O.D. 550 1.0 = 1.4 x 10^9 cells/ml, was placed in a 15 ml conical centrifuge tube, and spun down at 6000 g for 10 minutes. After centrifugation the supernatant was decanted and the tubes were held at room temperature until inoculum was needed, but not longer than one hour.

Inoculations were conducted in batches such that each plate of seed was treated with a newly resuspended pellet of *A. tumefaciens* harboring the 4X construct. One at a time the pellets were resuspended in 20 ml inoculation medium. Inoculation medium consisted of B5 salts (B5893), 3.2 gm/L; sucrose, 2.0% w/v; BAP, 44 μM; and indolebutyric acid (IBA), 0.5 μM. Acetosyringone (AS), 100 μM was added and the medium was buffered to pH 5.5 with MES, 10 mM.

The mixture was resuspended by vortexing and the inoculum was poured into a petri dish containing prepared seed and the cotyledonary nodes were macerated with a surgical blade. This was accomplished by dividing seed in half by longitudinal section through the shoot apex, preserving the two whole cotyledons. The two halves of the shoot apex were broken off at their respective cotyledons by prying them away with a surgical blade. The cotyledonary node was then macerated with the

surgical blade by repeated scoring along the axis of symmetry. Care was taken not to cut entirely through the explant to the abaxial side. Twenty explants were prepared in roughly five minutes and then incubated for 30 minutes at room temperature without agitation. Additional plates were prepared during this time. After 30 minutes the explants were transferred to plates of the same medium solidified with Gelrite (Merck & Co., Inc.), 0.2% w/v. Explants were embedded with the adaxial side up and level with the surface of the medium and cultured at 22°C for three days under cool white fluorescent light, approximately 20 μEm^2S^1.

After three days the explants were moved to liquid counterselection medium. Counterselection medium consisted of B4 salts (G5893), 3.2 gm/L; sucrose, 2.0% w/v; BAP, 5.0 μM; IBA, 0.5 μM; vancomycin, 200 μ/ml; cefotaxime, 500 μg/ml and was buffered to pH 5.7 with MES, 3 mM. Ten explants were washed in each petri dish with constant, slow gyratory agitation at room temperature for four days. Counterselection medium was replaced four times.

The explants were picked to agarose solidified selection medium. Selection medium consisted of B5 salts (G5893), 3.2 gm/L; sucrose, 2.0% w/v; BAP, 5.0 μM; IBA, 0.5 μM; kanamycin sulfate, 50 μg/ml; vancomycin, 100 μg/ml; cefotaxime, 30 μg/ml; timentin, 30 μg/ml and was buffered to pH 5.7 with MES, 3.0 mM. Selection medium was solidified with SeaKem agarose, 0.3% w/v. The explants were embedded in the medium, adaxial side down and cultured at 28°C with a 16 hour day length and cool white fluorescent light, of 60-80 μEm^2S^1.

After two weeks explants were washed with liquid medium on the gyratory shaker. This time the wash was conducted overnight in counterselection medium containing kanamycin sulfate, 50 μg/ml. The following day explants were picked to agarose solidified selection medium. Again they were embedded in the medium, adaxial side down, and cultured as before for another two weeks.

After one month on selective media transformed tissue became visible as green sectors of regenerating tissue against a background of bleached, less healthy tissue. Explants without green sectors were discarded, explants with green sectors were transferred to elongation medium. Elongation medium consisted of B5 salts (G5893), 3.2 gm/L; sucrose, 2.0% w/v; IBA, 3.3 μM; gibberellic acid, 1.7 μM; vancomycin, 100 μg/ml; cefotaxime, 30 μg/ml; timentin, 30 μg/ml and was buffered to pH 5.7 with MES, 3.0 mM. Elongation medium was solidified with gelrite, 0.2% w/v. Explants were embedded adaxial side up and culture as before. Culture was continued on this medium with transfers to fresh plates every two weeks.

When shoots became 0.5 cm in length they were excised at the base and placed in rooting medium in 13 x 100 mm test tubes. Rooting medium consisted of B5 salts (G5893), 3.2 gm/L; sucrose, 15 gm/L; nicotinic acid, 20 μM; pyroglutamic acid (PGA), 900 mg/L and IBA, 10 μM. The medium was buffered to pH 5.7 with MES, 3.0 mM and solidified with GelRite, 0.2% w/v. After ten days the shoots were transferred to the same medium without IBA or PGA. Shoots were rooted and held in these tubes under the same environmental conditions as before.

Once a root system was well established, the plantlet was transferred to sterile soil mix in plant cons (ICN Biomedicals, Inc.; catalogue Nos. 26-720 and 1-02). Temperature, photoperiod and light intensity remained the same as before. Under these conditions the regenerants became vigorous, somewhat small, but mostly normal plants. When their root systems became well established, a corner of the plant con was cut off and the plants were gradually hardened off in an environmental chamber or greenhouse. Finally they were potted in soil mix and grown to maturity, bearing seed, in a greenhouse.

C. Analysis of seeds produced by transgenic plants

Mature seed from several independent transgenic lines containing either the SFA-8 or 10 kDa rice prolamin genes were extracted in 2X Laemmli buffer. Proteins were quantitated using a modified Lowry kit from Bio-Rad using bovine serum albumin as a standard. Equal amounts of protein were loaded onto 4-20% Tris-glycine polyacrylamide gels. Molecular weight standards in the range of 3-116 kD were obtained from Novex and run alongside the protein samples. Gels were run in duplicate with one being electrophoretically transferred to Immobilon PVDF membrane for western blotting and the other being used for Coomassie staining. Western blots were performed using affinity purified antibodies against either the sunflower 2S albumin or rice prolamin and the signal was detected by the Western-Light Chemiluminescent Detection System from Tropix, Inc. (Bedford, MA) as per the manufacturer's instructions.

Total amino acid composition can be determined by acid hydrolysis of tobacco or soybean meal by standard protocols. Seed proteins also can be analyzed for trypsin inhibitory activity according to the previously described protocols of Kollipara *et al.*, *J. Agricul. Food Chem.* 40:2356 (1992). Similarly chymotrypsin inhibitory assays can be done according to Geiger, Chymotrypsin. In "Methods of Enzymatic Analysis," pp. 99-109 (1984).

Analysis of seed proteins from 6 independent transgenic tobacco lines that contain the gene for SFA-8 revealed a band that comigrated with purified SFA-8. No signal was detected in protein samples from non-transformed tobacco. By comparing the intensity of the immunodetected band to a dilution series of purified SFA-8, the transgenic SFA-8 protein accumulated as a percentage of total seed protein was calculated to be 5-10%. No larger precursor proteins were detected, indicating that the transgenic is processed as it normally is in sunflower.

Similar results were obtain for transgenic tobacco plants expressing the rice prolamin gene. A band was

detected that comigrated with the purified rice prolamin in all of the transgenic tobacco lines. No signal was detected in protein samples from non-transformed tobacco. Using a dilution series of purified rice prolamin as a standard, the transgenic rice prolamin protein accumulated as a percentage of total seed protein was calculated to be 1-5%. Surprisingly, no larger precursor proteins were detected, indicating that the transgenic tobacco plant, a dicot, processed the protein in the same way as rice, a monocot.

What Is Claimed Is:

1. A transgenic plant that contains a DNA molecule comprised of (A) a nucleotide sequence corresponding to a sulfur-rich rice prolamin gene or sulfur-rich sunflower 2S albumin gene and (B) a promoter, operably linked to said nucleotide sequence, to effect expression thereof by said transgenic plant.

2. A transgenic plant according to claim 1, wherein said plant is a soybean plant.

3. A transgenic plant according to claim 2, wherein said nucleotide sequence corresponds to a sulfur-rich 10 kDa rice prolamin gene.

4. A transgenic plant according to claim 3, wherein said nucleotide sequence is a sulfur-rich 10 kDa rice prolamin gene as shown in Figure 2.

5. A transgenic plant according to claim 2, wherein said nucleotide sequence corresponds to one of SFA-7 and SFA-8.

6. A transgenic plant according to claim 5, wherein said nucleotide sequence is SFA-8.

7. A transgenic plant according to claim 1, wherein said plant is a corn plant.

8. A seed produced by a plant as claimed in claim 1.

9. A method of increasing the content of sulfur-containing amino acids in an animal feed, comprising the steps of (i) providing seeds from a plurality of plants, at least some of which plants contain a DNA molecule comprised of a nucleotide sequence corresponding to a sulfur-rich rice prolamin gene or sulfur-rich sunflower 2S albumin gene, operably linked to a promoter to effect expression of said nucleotide sequence by said plants; and (ii) processing said seeds into animal feed.

10. A method according to claim 8, wherein said plants are soybean plants.

11. A method according to claim 10, wherein said nucleotide sequence corresponds to a sulfur-rich 10 kDa rice prolamin gene.

12. A method according to claim 9, wherein said nucleotide sequence corresponds to a sulfur-rich sunflower 2S albumin gene.

13. A feed product that contains soybean meal obtained from seed of a transgenic plant according to claim 1.

14. A feed product according to claim 13, wherein said feed product has not been supplemented by addition of sulfur-containing amino acids.

15. A method of altering a plant to produce seed that contains levels of sulfur-containing amino acids that are higher than those of an unaltered plant, comprising the steps of (i) providing cells or tissues of a plant transformed with an expression vector that contains a nucleotide sequence corresponding to a sulfur-rich rice prolamin gene or sulfur-rich sunflower 2S albumin gene; (ii) propagating plants from said transformed cells or tissue; and (iv) selecting for further propagation those plants that produce sulfur-rich rice prolamin or sulfur-rich sunflower 2S albumin.

Figure 1

ATG GCA AGG TTT TCG ATC GTG TTT GCA GCA GCA GGA GTA
Met Ala Arg Phe Ser Ile Val Phe Ala Ala Ala Gly Val
CTC CTC CTG GTA GCT ATG GCG CCA GTT TCT GAG GCT TCC
Leu Leu Leu Val Ala Met Ala Pro Val Ser Glu Ala Ser
ACC ACA ACC ATC ATC ACC ACC ATC ATA GAG GAG AAC CCC
Thr Thr Thr Ile Ile Thr Thr Ile Ile Glu Glu Asn Pro
TAT GGC CGA GGT AGA ACT GAA TCC GGA TGC TAT CAG CAG
Tyr Gly Arg Gly Arg Thr Glu Ser Gly Cys Tyr Gln Gln
ATG GAG GAG GCG GAG ATG CTC AAC CAC TGC GGG ATG TAC
Met Glu Glu Ala Glu Met Leu Asn His Cys Gly Met Tyr
CTC ATG AAA AAT TTA GGA GAA AGG AGC CAA GTT AGT CCC
Leu Met Lys Asn Leu Gly Glu Arg Ser Gln Val Ser Pro
AGA ATG AGA GAA GAG GAT CAC AAG CAA CTT TGC TGC ATG
Arg Met Arg Glu Glu Asp His Lys Gln Leu Cys Cys Met
CAA CTG AAG AAC CTA GAT GAG AAG TGC ATG TGT CCG GCA
Gln Leu Lys Asn Leu Asp Glu Lys Cys Met Cys Pro Ala
ATC ATG ATG ATG TTG AAC GAA CCA ATG TGG ATA AGG ATG
Ile Met Met Met Leu Asn Glu Pro Met Trp Ile Arg Met
CGA GAT CAA GTG ATG AGC ATG GCT CAT AAC CTG CCC ATA
Arg Asp Gln Val Met Ser Met Ala His Asn Leu Pro Ile
GAA TGC AAT CTC ATG TCC CAA CCA TGC CAA ATG TAA
Glu Cys Asn Leu Met Ser Gln Pro Cys Gln Met

Figure 2

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ATG GCA GCA TAC ACC AGC AAG ATC TTT GCC CTG TTT GCC
Met Ala Ala Tyr Thr Ser Lys Ile Phe Ala Leu Phe Ala
TTA ATT GCT CTT TCT GCA AGT GCC ACT ACT GCA ATC ACC
Leu Ile Ala Leu Ser Ala Ser Ala Thr Thr Ala Ile Thr
ACT ATG CAG TAT TTC CCA CCA ACA TTA GCT ATG GGC ACT
Thr Met Gln Tyr Phe Pro Pro Thr Leu Ala Met Gly Thr
ATG GAT CCG TGT AGG CAG TAC ATG ATG CAA ACG TTG GGC
Met Asp Pro Cys Arg Gln Tyr Met Met Gln Thr Leu Gly
ATG GGT AGC TCC ACA GCC ATG TTC ATG TCG CAG CCA ATG
Met Gly Ser Ser Thr Ala Met Phe Met Ser Gln Pro Met
GCG CTC CTG CAG CAG CAA TGT TGC ATG CAG CTA CAA GGC
Ala Leu Leu Gln Gln Gln Cys Cys Met Gln Leu Gln Gly
ATG ATG CCT CAG TGC CAC TGT GGC ACC AGT TGC CAG ATG
Met Met Pro Gln Cys His Cys Gly Thr Ser Cys Gln Met
ATG CAG AGC ATG CAA CAA GTT ATT TGT GCT GGA CTC GGG
Met Gln Ser Met Gln Gln Val Ile Cys Ala Gly Leu Gly
CAG CAG CAG ATG ATG AAG ATG GCG ATG CAG ATG CCA TAC
Gln Gln Gln Met Met Lys Met Ala Met Gln Met Pro Tyr
ATG TGC AAC ATG GCC CCT GTC AAC TTC CAA CTC TCT TCC
Met Cys Asn Met Ala Pro Val Asn Phe Gln Leu Ser Ser
TGT GGT TGT TGT TGA
Cys Gly Cys Cys ●●●
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INTERNATIONAL SEARCH REPORT

Inter. ...nal Application No
PCT/US 97/06180

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C07K14/415 //A01H5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PATENT ABSTRACTS OF JAPAN vol. 095, no. 011, 26 December 1995 & JP 07 213185 A (SUMITOMO CHEM CO LTD), 15 August 1995, see abstract ---	1-4, 7-11, 13-15
A	WO 92 14822 A (E.I. DU PONT DE NEMOURS AND COMPANY) 3 September 1992 see page 2, line 30 - page 4, line 5 ---	1-15
A	MASUMURA T ET AL: "CDNA CLONING OF AN MRNA ENCODING A SULFUR-RICH 10 KDA PROLAMIN POLYPEPTIDE IN RICE SEEDS" PLANT MOLECULAR BIOLOGY., vol. 12, February 1989, DORDRECHT NL, pages 123-130, XP000045137 cited in the application see the whole document ---	1,3,4,9, 11,13,15
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
5 February 1998	23.02.98
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	**Authorized officer** De Kok, A

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/US 97/06180

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	L.M. TABE ET AL: "A biotechnological approach to improving the nutritive value of alfalfa - sunflower seed albumin sulfur-rich protein gene expression in transgenic alfalfa" THE JOURNAL OF ANIMAL SCIENCES, vol. 73, no. 9, 1995, CHAMPAIN US, pages 2752-2759, XP002054593 see the whole document	1,5,6,9, 12
A	A.A. KORTT ET AL: "Amino acid and cDNA sequences of a methionine-rich 2S protein from sunflower seed (Helianthus annuus L.)" THE EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 195, 1991, BERLIN DE, pages 329-334, XP002054594 see the whole document	5,6,12
A	EP 0 318 341 A (PLANT GENETIC SYSTEMS NV) 31 May 1989 see the whole document, especially page 13, Table 1	1,5,6,12
A	EP 0 295 959 A (PLANT CELL RESEARCH INSTITUTE, INC.) 21 December 1988	1-15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/06180

Box I Observation where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1,2,7-10,13-15 partially and 3,4, and 11 completely

A transgenic plant that contains a DNA molecule comprised of (A) a nucleotide sequence corresponding to a sulfur-rich rice prolamin gene and (B) a promoter, and its use in the production of animal feed.

2. Claims: 1,2,7-10,13-15 partially and 5,6 and 12 completely

A transgenic plant that contains a DNA molecule comprised of (A) a nucleotide sequence corresponding to a sulfur-rich sunflower 2S albumin gene and (B) a promoter, and its use in the production of animal feed.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte. .ional Application No
PCT/US 97/06180

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